RIBULOSE DIPHOSPHATE CARBOXYLASE ACTIVITIES IN COLD-RESISTANT COMMON MALLOW, *MALVA NEGLECTA* WALLR. AND A COLD-SENSITIVE TOMATO, *LYCOPERSICON ESCULENTUM* L., ACE 55 VAR.

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Abstract.— Common mallow (*Malva neglecta* Wallr.) and tomato (*Lycopersicon esculentum* L. var. Ace 55) were compared as to certain characteristics: Co_2 fixation properties, ribulose diphosphate carboxyl activities, (RuDPCase) photosynthesis, respiration, and compensation points. Significant differences in these factors were observed in all cases except dark respiration. Mallow enzyme (RuDPCase) activities were higher per unit of enzyme than those of tomato. The Mallow RuDPCase exhibited slightly higher activity at 5 to 25 C. Mallow leaves retained their capacity for photosynthesis and respiration after long periods of exposure to subfreezing temperature. The cold adapted mallow had a higher CO_2 compensation point, suggesting a lower efficiency for CO_2 fixation. The results suggest that cold acclimation in common mallow affects photosynthesis but has little effect on respiration.

Several physiological factors are associated with the development of resistance to winter injury in plants. Qualitative and quantitative changes in protein, carbohydrate, and lipid contents have been observed during cold acclimation (Roberts 1969, Gerloff et al. 1967, Hochachka and Somaro 1968, Zeller 1951). However, the *in vivo* features of observed biochemical and physiological alterations associated with cold acclimation in specific instances are not clear. In particular, very little is known about the intracellular mechanisms of freezing resistance in broad-leaved plants that remain conspicuously green and metabolically active throughout the winter months of cool temperature regions.

Common mallow, *Malva neglecta* Wallr., is an example of a broad-leaved plant that often remains green and succulent throughout the winter in north-temperature regions. Its green leaves can tolerate subfreezing temperatures without visible evidence of injury. It appears that common mallow is capable of surviving winter cold by some mechanism other than dormancy, because the plant retains the capacity for photosynthesis and relatively high respiration rates when favorable conditions are present.

These observations have prompted an investigation of certain photosynthetic characteristics and CO_2 fixation properties in winter-hardened mallow. This paper reports the activity of purified ribulose diphosphate carboxylase (RuDPCase) and the capacity of whole leaves to fix CO_2 from cold-acclimated, field-grown mallow and from greenhouse-grown mallow and tomato.

Materials and Methods

Plant materials: Common mallow is a perennial weed characteristic of cultivated ground, gardens, yards, and waste places throughout the United States. Introduced from Europe, the weed belongs to the same plant family as cotton, hollyhocks, rose of sharon, and the weeds known as velvet-leaf and flower-of-the-hour. This family (Malvaceae) has flowers which contain a tube of stamens surrounding the pistil and a ring of seeds centered in persistent floral parts reminiscent of a small flat cheese (thus one of the plant's common names, "cheese weed"). The plant's long tap root and its wide distribution in relation to habitat occupation indicates a wide ecological amplitude in regard to environmental stress factors.

The garden tomato, *Lycopersicon esculentum* L., variety Ace 55, cannot tolerate subfreezing temperatures. Tomatoes are warm

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season plants; the Ace 55 variety yields very well under high day and cool night temperature regimes.

Method of sampling plants for measurement of photosynthesis and respiration rates: Plants of mallow and tomato were grown in the greenhouse at 25 C (76 F) day and 20 C (68 F) night temperatures. Mallow plants were also grown in the field near Provo, where they were exposed to subfreezing temperatures. Plant samples were taken from greenhouse and field areas during January. When harvested, plants were collected whole, petiole ends were cut under water, and then they were placed in controlled environment chambers with the cut ends remaining immersed in water. Only deep green succulent growth was harvested. Photosynthetic and respiration measurements were then made repeatedly as described below.

CO_2 fixation methods: Rates of net photosynthesis (APS), dark respiration (DR), and CP_2 compensation points (CP) were determined in mid-January on excised shoots cut under water. Analysis was made utilizing a Beckman IR-215 infrared gas analyzer and a plexiglas controlled-environment chamber. Apparent photosynthesis (APS) was determined by the time required for the closed system's CO_2 content to decrease from 315 to 275 μl per l of air. Dark respiration (DR) was determined by the time for the closed system's CO_2 content to return from 275 to 315 μl per l. The CO_2 compensation point (CP) was determined in a closed system by allowing the plants to fix CO_2 until no further change in CO_2 concentration occurred in the atmosphere of the lighted plexiglas chamber surrounding the plant. The assimilation chamber was housed inside a large growth chamber with lighting provided by 8 cool-white inforescent tubes, 8 grolux (Sylvania) inforescent tubes, and 10 25-watt incandescent globes. The light was filtered through 4 cm of water and provided an intensity of 6.05 × 104 ergs per cm per minute at leaf height. This light intensity has been indicated to be saturating for tomato at 315 μl CO_2 per l of air. Chamber parameters were: temperature –23 ± .6 C; relative humidity—65 ± 10 percent; wind speed—4.0 dm per minute (3 chamber volumes per minute) (Brewster 1971).

Preparation of extracts from leaf homogenates and enzyme purification: Fully expanded leaves were washed and their midribs removed and blotted dry. From this step on, all procedures were carried out at 5 C. Approximately 3.0 gm samples of leaf tissue were ground manually with cold mortar and pestle for 5 minutes, using 5 ml of 0.1 M HEPES (N-2-hydroxyethylpiperzaine-N-2-ethanesulfonic acid) buffer pH 8.00, 0.001 M EDTA, 0.0001 M DTT, 0.01 M $MgCl_{21}$, 0.025 mM NaHCO3, per gram fresh weight leaf tissue. The homgenates were centrifuged for 10 minutes at 20,000 rpm in a Sorvall model RC-2B centrifuge with the S-34 rotor. The supernatant was decanted and used as the crude enzyme extract. The crude enzyme extract from the low speed centrifugation was further clarified by centrifugation at 40,000 rpm in a Spinco model L3-50 for 5 minutes. The supernatant fraction was collected. The RuDPCase enzyme was purified further by sedimentation of the extract into a sucrose step gradient consisting of 2 ml of 5 percent, 2 ml 30 percent and 3 ml 50 percent sucrose solution in HEPES buffer layered in a centrifuge tube. The rapidly sedimenting RuDPCase accumulated in the 50 percent sucrose layer after sedimentation for 12 hours at 25,000 rpm in a Spinco SW-25 rotor. The sucrose-enzyme solution was then passed through a 10 × 1.0 cm Sephadex G-25 column for further purification.

Measurement of enzyme activities: Determination of RuDPCase activity was based upon fixation of $^{14}CO_2$- into acid stable products. The assay mixture contained 0.01 M HEPES-SO4 buffer (pH 8.00) 0.01 M MgCl2, 0.001 M DTT, 0.02 M $NaH^{14}CO_3$, and ribulose-1, 5-bisphosphate in 200 μl. The enzyme (30μl) was added to initiate the reaction and was allowed to proceed for 10 minutes. The reaction rates were linear over this time period. The enzyme reaction was stopped by the addition of 50 μl of glacial acetic acid. A 100 μl aliquot of the reaction mixture was spotted onto a strip of Whatman No. 1 filter paper and dried under the hood. The sample was counted in a Packard Tri-Carb (Model 3320) liquid scintillation counter in toluene scintillation fluid. The counted samples were corrected for machine efficiency and quenching

and the values converted to disintegrations per minute (dpm).

Determination of specific activity of RuDPCase: RuDPCase has been identified with a large, rapidly moving boundary observed in the Spinco Model E ultracentrifuge known as fraction I protein. The area of the Schlieren boundary curve corresponding to fraction I protein provides a means to determine the relative concentration of RuDPCase present. An estimate of specific activity per unit of enzyme can then be calculated by comparing enzyme activity in a given extract with the area of the corresponding fraction I protein peak (Andersen et al. 1970).

RESULTS

Net photosynthesis, dark respiration, CO_2 compensation point: Table 1 shows that cold-adapted, field-grown mallow exhibited lower rates of APS than greenhouse-grown mallow or tomato. However, rates of DR were similar in all cases. This resulted in an APS to DR ratio for field-grown mallow of one-half that for the greenhouse-grown plants. The CP shows a significant increase for field-grown Mallow over values for the greenhouse-grown plants.

Reaction velocities for carbonate and ribulosediphosphate substrates: The comparative reaction velocities for purified "cold-adapted" mallow and tomato RuDPCase at different carbonate substrate concentrations are depicted in Figure 1. The ribulosediphosphate (RuDP) substrate was maintained at maximum concentration for both enzymes. The mallow RuDPCase exhibited higher catalytic capacity per unit of enzyme than tomato RuDPCase. The turnover number at V_{max} (4 u moles of carbonate substrate per 200 ul of reaction mixture) for tomato (RuDPCase) was calculated at 1036 moles carbonate fixed per mole of enzyme per minute. The turnover number for puri-

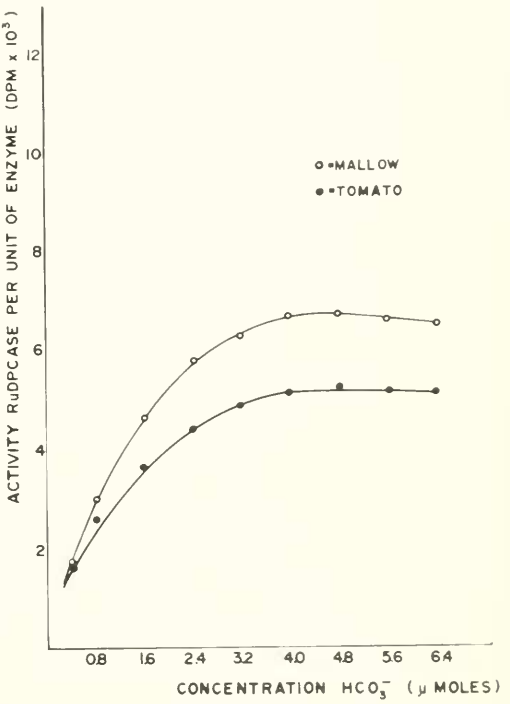

Fig. 1. Dependence of RuDPCase activity in purified extracts upon HCO3 concentrations. RuDPCase was purified from common mallow and tomato ACE 55 var. The enzyme activities are based upon total amount of RuDPCase enzyme present in the reaction mixture as calculated from the Schlieren curve of the sedimenting boundaries in a model E ultracentrifuge.

TABLE 1. Rates of net photosynthesis, dark respiration, and the CO_2 compensation point of excised plant shoots. See text for description of plant treatments.

Species	Location	[a] Apparent photosynthesis ($ugCo_2 \cdot dm^{-2} \cdot min^{-1}$)	[b] Dark respiration ($ugCo_2 \cdot dm^{-2} \cdot min^{-1}$)	[c] CO_2 compensation point ($ul.l^{-1}$)
Mallow	Greenhouse	3.2	1.7	64
Mallow	Field	1.5	1.7	86
Tomato (ACE 55)	Greenhouse	2.6	1.3	68

[a]Apparent photosynthesis (APS) was measured by determining time required to lower closed system CO_2 concentration from 320 ul/l to 280 ul/l air-l.
[b]Dark respiration (DR) was measured by determining the time required for a darkened closed system to return CO_2 concentration from 280 to 320 ul/l air.
[c]Compensation point was measured by allowing photosynthesizing plants to fix CO_2 from a closed atmosphere until no further change in CO_2 concentration could be observed.

fied mallow RuDPCase was calculated at 1400 moles of carbonate fixed per mole enzyme per minute. The differences in V_{max} values for the two enzymes were judged to be highly significant, based on the student-t test for measuring differences between paired variates. The calculated t value exceeded the 0.001 level of significance. Only slight differences between the corresponding Km values could be observed.

The reaction velocities of mallow and tomato RuDPCase for different ribulosediphosphate substrate concentrations are graphed in Figure 2. The shapes of the reaction velocity curves for tomato and Mallow RuDPCse are similar. The mallow enzyme exhibited a significantly higher V_{max} value. Both enzymes showed substrate inhibition at RuDP substate concentrations higher than 8 u moles per 200 µl of reaction mixture. The significance of the differences between the two reaction velocity curves in Figure 2 was measured by the student-t test for paired variates. The calculated t value exceeded the 0.01 level of significance. The Km values for substrate concentration at half maximal velocity were slightly higher for tomato.

Effect of temperature on reaction velocity with purified RuDPCase enzyme: Purified tomato and mallow RuDPCase enzyme extracts were compared for catalytic velocities at reaction temperatures ranging from 4 C to 63 C (Figure 3). Purified mallow RuDPCase had significantly higher catalytic activity per unit of enzyme under the temperature range of 4 to 25 C. The calculated student-t value for differences between paired variates exceeded the 0001 level of significance. On the other hand, purified tomato enzyme showed significantly higher catalytic capacity in the 38 to 53 C temperature range. The corresponding calculated student-t value for paired reaction rates in the 38 to 53 C temperature range exceeded the 0.01 significance level. The general shapes of the temperature curves for the purified mallow and tomato RuDPCase were quite similar, however, with heat denaturation for both enzymes occurring near 53 C.

Discussion

Our studies indicate that respiration and photosynthesis are differentially effected by

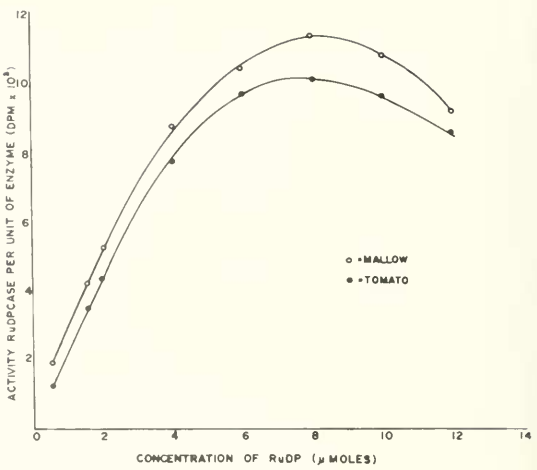

Fig. 2. Dependence of RuDPCase activity in purified extracts from mallow and tomato ACE 55 var. upon RuDP concentrations. Enzyme activities are normalized for equal concentrations of RuDPCase.

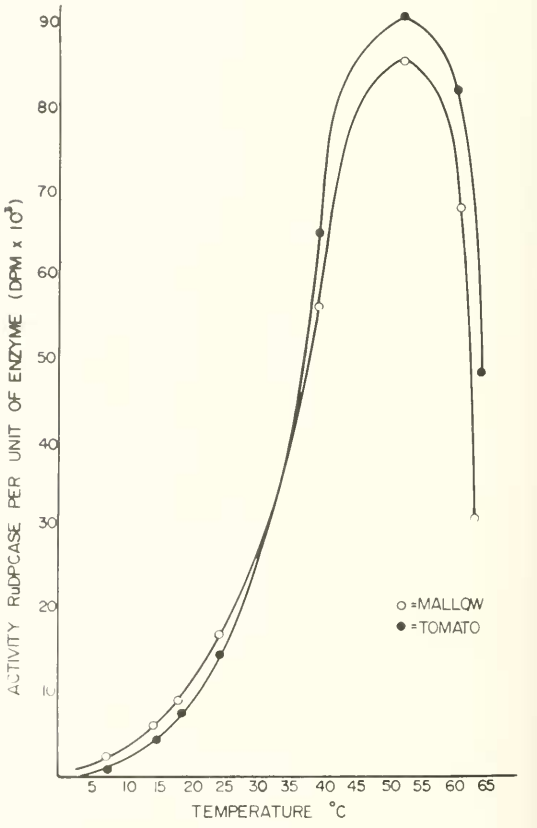

Fig. 3. Dependence of RuDPCase activity in purified extracts from mallow and tomato ACE 55 var. upon temperature. Enzyme activities are normalized for equal concentrations of RuDPCase.

cold acclimation in mallow. The measured rates of apparent photosynthesis values in greenhouse-grown mallow were approximately twice the measured rate of apparent photosynthesis values of cold-acclimated, field-grown mallow. No differences were observed for rates of dark respiration (Table 1). Higher CO_2 compensation points were observed for field-grown, cold-adapted mallow, which suggested a depressed efficiency for CO_2 fixation. However, because respiration and photosynthesis measurements were made at 25 C in the laboratory, it is possible that relative efficiencies of carbohydrate accumulation would change at lower temperatures. The cold-acclimated mallow might under such circumstances become relatively more efficient. Present evidence, however, indicates that cold acclimation in field-grown mallow is a matter of maintaining a steady state of metabolic activity rather than the rapid accumulation of carbohydrate reserves. Further studies are underway to assess interaction of lower temperatures and carbohydrate accumulation in cold-acclimated mallow.

Purified RuDPCase from cold-adapted *Malva neglecta* had the highest catalytic capacity per unit of enzyme. The Vmax values for carbonate and ribulosediphosphate substrates were highest for mallow RuDPCase (Figs. 2 and 3). The Km values for tomato were only slightly higher. The Km and Vmax values for tomato RuDPCase agree in general with corresponding reported values (Andersen et al. 1970). Although we cannot yet compare *in vitro* RuDPCase activity to *in vivo* CO_2 fixation without some misgivings, our studies suggest that mallow RuDPCase would promote slightly more rapid CO_2 fixation per unit of enzyme *in vivo*. The lower Km value for CO_2 substrate of RuDPCase from the cold-adapted mallow would suggest a higher photosynthetic efficiency for the intact plant. Yet the higher compensation point of these cold-adapted plants indicates that photosynthetic efficiency is depressed in the intact leaf. Recent work by several investigators clearly implicates RuDPCase as a major contributing factor to the high compensation points of the C3 species (Ogren and Hunt 1978). Our study would indicate that the higher compensation point in the cold-adapted mallow is due to some other factor in the photosynthetic carbon cycle than RuDPCase. On the other hand, since we are using purified enzyme for our studies, it is likely that control molecules that may affect Km for CO_2 fixation of RuDPCase could be removed in our purification process. In any case, if RuDPCase has a higher Km for CO_2 *in vitro*, which would result in a higher compensation point in the cold-adapted mallow, the effect does not persist through purification of the enzyme. Therefore, at least a change has not been detected on the purified enzyme that would affect the compensation point and thus be a basis for lower photosynthetic efficiency during cold acclimation.

The mallow RuDPCase enzyme showed higher catalytic capacity than tomato RuDPCase under temperature ranges of 0–25 C, and the tomato RuDPCase enzyme exhibited higher activity under temperature ranges 40–60 C. This may be indicative of Mallow's lower-temperature environmental adaptation and its CO_2-fixing enzymes. Other studies have shown that RuDPCase extracts from plants of different climatic regions exhibit correspondingly different temperature reactions (Triharne and Cooper 1969). Also, we have observed that the RuDPCase activities in crude extracts from Mallow were consistently higher than RuDPCase activities in tomato crude extracts, (on a per-gram fresh weight basis). These results, along with the distribution patterns of these two species, suggest that the temperature interaction of the enzyme might be related in some way to the different seasonal adaptations of the two species.

The results also suggest that the process of cold acclimation in mallow affects photosynthesis and dark respiration differently. Respiration was not seriously affected, but photosynthetic capacity per unit of leaf area and photosynthetic efficiency were significantly reduced (Table 1). It may be hypothesized then that the processes of cold acclimation in mallow either depresses the *in vivo* activity of RuDPCase or alters in some way other chloroplast functions which affect the plant's capacity for photosynthesis.

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LITERATURE CITED

ANDERSEN, W. R., G. F. WILDNER, AND R. S. CRIDDLE. 1970. Ribulose diphosphate carboxylase from mutant tomato plants. Arch. Biochem. Biophys. 137:84–90.

BREWSTER, S. F. 1971. The physiological vitality of scarlet globemallow, *Sphaeralcea grossulariaefolia* (Hook. & ARN.) Rydberg, under drought. Unpublished dissertation, Brigham Young University, Provo, Utah.

GERLOFF, E. D., M. A. STAHMANN, AND D. SMITH. 1967. Soluble proteins in alfalfa roots as related to cold hardiness. Plant Physiol. 42:895–899.

HOCHACHKA, P. W., AND G. N. SOMERO. 1968. Adaptations of enzymes to temperatures. Comp. Biochem. Physiol. 27:659–668.

OGREN, W. L., AND L. D. HUNT. 1978. Comparative biochemistry of ribulose-bisphosphate carboxylase n higher plants. Pages 127–139 *in* Siegelman and Hind, eds., Photosynthetic carbon assimilation. Basic Life Sciences 11.

ROBERTS, D. W. A. 1969. A comparison of isozymes of wheat plants grown at 6 C and 20 C. Canadian J. Bot. 47:263–265.

TREHARNE, K. J., AND J. P. COOPER. 1969. Effect of temperature on the activity of carboxylase in tropical and temperature graminae. J. Exp. Bot. 20:170–175.

ZELLER, O. 1951. Respiration at low temperatures in cold hardened cereals. Planta. 39:500.